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ABSTRACT

Human neural progenitors have a strong potential for use as cell-based biosensors for environmental toxins. In this report, we demonstrate that we have (1) optimized methods to genetically modify hNP1™ cells, (2) devised strategies for enriching the neural progenitor cell population using cell surface markers, and (3) developed methods for directed dopaminergic differentiation using defined medium conditions – all towards the goal of accelerating neuronal differentiation for biosensor development. Moreover, we have begun an exploration of fluorescence-based assays as a new direction for 'sensor element' development, using neurite outgrowth as a test platform for screening neurotoxicants. Such exploration of alternate 'sensor elements' beyond microelectrode arrays will broaden the utility of a human neural cell-based biosensor.

OUTLINE:

- OVERVIEW
- SYNOPSIS OF PREVIOUS RESULTS (Q1 2010)
- CURRENT RESULTS (Q2 2010)
- SUMMARY
- RECENT PUBLICATIONS &PATENTS
- REFERENCES

OVERVIEW

Effective monitoring of environmental toxicants remains a major challenge of great importance for both military and civilian populations, as existing technologies assess only known toxicants and are limited in scope. To address this concern, human-based biosensors are being developed to detect known and unknown toxicants. Prototype biosensors used mammalian neural networks on microelectrode array (MEA) sensor elements. However, these prototypes used primary cultures of neurons with limited life spans (approximately 2 weeks in the field), making frequent replacement a necessity. Thus, a field worthy biosensor will require a ready source of long-lived neurons. ArunA Biomedical has developed a robust human neural progenitor (hNP1™) cell lines from NIH approved human embryonic stem cell lines.

This project aims to develop techniques to improve and accelerate human neural progenitor cell differentiation into functional neural networks for use in human-based biosensors with MEAs and fluorescence-based assays as sensor elements. Optimizing the formation of stable, functional networks will be required to develop an efficient human-based biosensor device. To that end, we are taking several approaches: (1) genetic modification of existing human neural progenitor cells to accelerate differentiation into neurons, (2) cell sorting via neuron-specific carbohydrate epitopes and cell surface receptors to enrich for differentiated cells, and (3) use of support cells, growth factors, and steroids as a means to promote neuronal survival and network formation. In addition, difficulties in culturing and forming functional neural networks on MEAs have prompted a change in direction for development of the 'sensor element.' As an alternate approach, we are currently exploring the use of fluorescence based assays as a means to monitor neural cell health, function and viability.

Progress to date on these approaches is presented below.

SYNOPSIS OF PREVIOUS RESULTS (Q1 2010)

Overexpression of bHLH transcription factors

Expression of bHLH transcription factors pre- and post-differentiation by RT-PCR

Expression of endogenous bHLH transcription factors, Neurogenin 1, Neurogenin2, Neurogenin3 and NeuroD1 were upregulated upon withdrawal of FGF2, as expected during the differentiation of hNP1™ into neurons. This result suggested that the overexpression of bHLH transcription factors may accelerate neuron differentiation.

Transduction studies of human neural progenitor cells using various vectors

We also assessed the use of lentiviral transduction in ArunA's hNP1™ cells as a means to overexpress bHLH transcription factors. We observed that ArunA's hNP1™ cells were efficiently transduced with VSV-G pseudotyped lentiviral vectors with either the CMV or EF1α promoter and will be used in upcoming studies.

Attempts to improve single-cell colony formation using ROCK inhibitors

In our initial studies, we observed that culturing human neural progenitor cells below a plating density of 7,800 cells/cm^2 severely and irreversibly compromised proliferation and viability. Previous studies have shown that treatment with a highly potent, cell-permeable selective inhibitor of Rho-associated protein kinase (ROCK inhibitor), can enhance the cloning efficiency of dissociated human embryonic stem cells (hESCs) without affecting their pluripotency (Watanabe, et al., 2007). Thus, we hypothesized that ROCK inhibitor treatment of human neural progenitors might assist in the generation of clonal human neural progenitor cell lines. We observed that human neural progenitor cells seeded at low densities showed substantial proliferation in the presence of 10 μM ROCK inhibitor for up to 10 days. We are continuing to test ROCK inhibitor as a potential way to generate single cell clones by serial dilution.

Enrichment of mature neural cells using cell surface markers

In addition to using bHLH overexpression, we also explored whether a positive selection strategy to enrich for neural progenitors that readily differentiate can also improve and accelerate formation of mature neural networks. To that end, we examined cell surface markers in hESC-derived neural progenitors.

Changes in carbohydrate epitopes (i.e., lectin binding) upon neural differentiation

Carbohydrates corresponding to PhaL, VVA, DBA, LTL and PNA lectin binding are up regulated upon differentiation of hESCs into neural progenitors. In particular, VVA, DBA, LTL and PNA lectin binding increases in human neural progenitor cells but not in human mesenchymal stem cells (hMPCs). This suggests that these carbohydrates might be specific for human neural progenitor cell function and could be used for enrichment of human neural progenitor cells from a mixed population of cells. In future work VVA and PNA lectins will be used to isolate a pure population of human neural progenitor cells and used for developing a neural cell based biosensor.

Changes in cell surface receptors (i.e., CNTFRα) upon neural differentiation

Our preliminary high throughput cell membrane proteomic screening and flow cytometry studies identified ciliary neurotrophic factor receptor alpha (CNTFRα) as a potential, novel cell surface marker. CNTFRα has been implicated in regulating neural proliferation and lineage differentiation (Davis, et al., 1991; Ozog, et al., 2008). We found that 73% of ArunA's hNP1™ cells were CNTFRα positive compared to 1% for the WA09 hESC parent line. We hypothesize that the CNTFRα positive neural progenitors will show enhanced capacity for neural differentiation. In future studies we plan to determine the

differentiation potential of both CNTFRα-positive and -negative populations and use the population with enhanced differentiation potential for genetic modification. By combining both CNTFRα selection and genetic modification, we hope to further expedite the rapid formation of electrically active and functional human neural networks.

CURRENT RESULTS (Q2 2010)

Use of defined media conditions to accelerate differentiation and synaptic protein expression
Use of GDNF to promote dopaminergic differentiation

Rationale

Human neural progenitor cells derived from hESCs have demonstrated an ability to produce various cell types. The objective of this study was to determine whether previously ArunA's hNP1™ cell lines could be differentiated into dopaminergic neurons using GDNF, a glial cell line derived neurotrophic factor known to promote the survival of dopaminergic neurons (Lin, et al., 1993). Directed differentiation using defined factors, such as GDNF, may help to accelerate the formation of mature neural networks. Moreover, human neural progenitor cells expressed the GDNF receptor RET and the transcription factor NURR1, both of which are involved in dopaminergic differentiation (e.g., Galleguillos, et al., 2010). This result suggests that ArunA's hNP1™ cells are amenable to dopaminergic differentiation using treatment with GDNF.

Methods

Human neural progenitor cell culture and maintenance: Human neural progenitor cells were propagated in proliferation medium (containing bFGF). Media were changed every other day and cells were passed every fourth day or as needed. Cells used for this experiment were passage 22-39.

Dopaminergic Neural Differentiation: Human neural progenitor cells were grown on polyornithine/laminin coated 35mm plates (Falcon) for flow cytometry or on polyornithine/laminin coated 4 well slides (Falcon) for immunocytochemistry staining. Cells were plated in proliferation medium. At 24 hours post-plating, the medium was changed to neural differentiation medium (proliferation medium without bFGF) plus or minus 25 ng/ml GDNF (Neuromics). Medium were changed every three days. Cells were harvested at differentiation day 0, 3, 7, 14 and 21 for further analysis.

Immunocytochemistry and Cell Quantification: Cells were fixed with 2% paraformaldehyde, blocked in 6% goat serum, then stained with primary and fluorophore-tagged secondary antibodies. Stained cells were visualized using spinning disk confocal microscope (Olympus). Cell counting was performed using Image Pro software (Media Cybernetics). Five random visual fields were selected and counted in triplicate. Data are presented as mean ±SD. Values of $p<.05$ was considered significant using ANOVA and Tukey's Pair-Wise test (Statistical Analysis Software, SAS Institute).

Flow Cytometry: Cells were fixed with 4% PFA, blocked in 6% goat serum, then stained with primary and fluorophore-tagged secondary antibodies. Levels of fluorescence in stained cells were measured on Dako Cyan flow cytometer(Beckman Coulter). Negative controls were secondary only and cell only staining. Data analysis was done using FlowJo (TreeStar) software. Each experiment was run in triplicate. Data are presented as mean ±SD. Values of $p<.05$ was considered significant using ANOVA and Tukey's Pair-Wise (Statistical Analysis Software, SAS Institute).

Results

During the differentiation process (withdrawal of bFGF), the addition of GDNF to human neural progenitor cells greatly increased the number of tyrosine hydroxylase (TH) positive cells after 21 days in culture, as measured by flow cytometry (51% positive with GDNF compared to 3% without; Table 1). TH, the rate limiting enzyme for dopamine synthesis, is a frequently used marker for dopaminergic

differentiation. At day 21 of differentiation, only cells treated with GDNF expressed PITX3 (Table 1), a transcription factor expressed specifically in midbrain dopaminergic neurons and required for their survival (Smidt, et al., 2004). At day 14, DAT expression significantly increased ($p<.05$) at day 14 in the treated cells (16.5 ± 3.1) compared with neurons differentiated without GDNF (5.4 ± 1.7; Figure 4C). The marked increase in expression of dopaminergic neuronal markers in human neural progenitor cells differentiated with GDNF over those without (Table 1) indicate a robust drive toward dopaminergic differentiation using the growth factor.

As a control for the flow cytometric results, we examined marker localization in differentiated cells by immunocytochemistry (Figure 1). human neural progenitor cells differentiated with GDNF for 21 days expressed TH (Figure 1, *middle panel*; Table 1) and PITX3 (Figure 1, *right panel*). Similar to previous studies, localization of TH and DAT (data not shown) was cytoplasmic, while PITX3 localization was nuclear (Messmer et al. 2007; Smidt and Burbach 2007).

Here we demonstrate a novel method for deriving dopaminergic neurons from a starting human neural progenitor cell population through a one-step differentiation process without the use of feeder cells. In this study, the derivation of dopaminergic-like cells also allowed for a shorter time to differentiation than previous reports using hESC as the starting population (Perrier et al. 2004), hence providing a tool to accelerate neural network formation. Previously, hESCs had been differentiated to dopaminergic neurons using complex, time-consuming approaches, such as the five-stage method or the SDIA method (Perrier, et al., 2004; Yan, et al., 2005). In summary, our results suggest that ArunA's hNP1™ cells are primed to become dopaminergic neurons and that the administration of GDNF promotes dopaminergic differentiation.

		Percentage of positive cells		
Marker	**Description**	***GDNF-treated***	***Untreated***	**Day post-differentiation**
EN1	transcription factor	74 ± 1 %	38 ± 6 %	day 21
TH	tyrosine hydroxylase	51 ± 2 %	3 ± 0 %	day 21
DAT	dopamine transporter	17 ± 3 %	5 ± 2 %	day 14
PITX3	transcription factor	66 ± 2 %	0 ± 0 %	day 21

Table 1. Flow cytometric results for human neural progenitor cells differentiated via withdrawal of bFGF in the presence or absence of 25 ng/ml GDNF. All differences shown were statistically significant ($p < 0.5$).

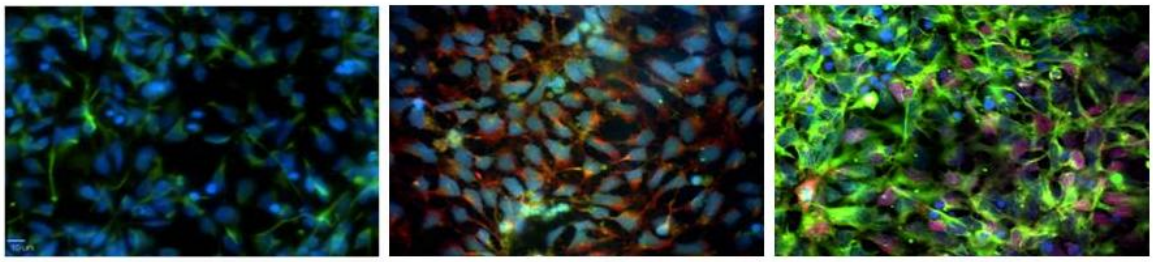

Figure 1. Immunofluorescence for human neural progenitor cells differentiated in the presence of 25 ng/ml GDNF. Cells were also counterstained for nuclei (DAPI) and the neuronal marker Tuj (β III-tubulin).

Development of sensor elements

Rationale

Use of fluorescence based assays to monitor neuronal health and viability

The initial direction for the project was to use microelectrode arrays (MEAs) as 'sensor elements' for a neural cell-based sensor. Difficulties in culturing and forming functional neural networks on MEAs have prompted a change in direction for development of the 'sensor element.' Currently, we are exploring the use of fluorescence based assays as a means to monitor neural cell health, function and viability. In a high-throughput format, these assays can be used as versatile sensors to screen a broad variety of environmental toxins rapidly and efficiently. In collaboration with William Mundy's group at the EPA, we conducted a study looking at neurite outgrowth as a method to monitor neurotoxicity.

Exposure to neurotoxic compounds during development can potentially cause nervous system defects and functional deficits throughout life (Rice & Barone, 2000; Costa, et al., 2004; Grandjean & Landrigan, 2006). Neurite outgrowth, a critical aspect of neural development, can be recapitulated using *in vitro* models, making them valuable tools for studying the molecular mechanisms that underlie the process (Zhang, W, et al, 2009; Zhang, Y, et al., 2009; Yu & Malenka, 2003; Redmond, et al., 2002; Jin, et al., 2003; Khaibullina, et al., 2004). These models can also be used to investigate the mechanism(s)-of-action for known developmental neurotoxicants (Yamauchi, et al., 2007; Lein, P., et al., 2000; Howard, et al., 2005; Audesirk, et al., 1991). Configured as high-throughput screening assays, *in vitro* measures of neurite outgrowth can provide a way to identify and prioritize potential developmental neurotoxicants (Radio, et al., 2008a; Radio, et al., 2009; Radio, et al., 2008b; NRC, 2007; Coecke, et al., 2007; Lein, et al., 2005). Here, we examined differentiated neural progenitors, hN2^{TM}, as an *in vitro* model for neurite outgrowth using immunocytochemical staining and high content image analysis (HCA). Our results demonstrate that select agents known to inhibit neurite outgrowth in rodent models decreased neurite outgrowth in hN2^{TM} cells. These results are detailed in a recent publication in *Neurotoxicology* (Harrill, et al., 2010).

Methods

Cell Culture: Costar® 96-well polystyrene cell culture dishes (Corning, Inc., Corning, NY) were coated poly-L-lysine then with laminin. hN2^{TM} cells were stored at -170°C and thawed at time of use. After thawing at 37°C, cells were suspended in serum-free ArunA basal medium (ABS^{TM}) supplemented with ArunA Neural Supplement (ANS^{TM}), leukemia inhibitory factor (LIF, 10 ng/mL), penicillin (50 U/mL), streptomycin (50 7 µg/mL) and 2 mM L-glutamine. Live cell yields post-thawing ranged from 60 to 80%.

Chemical Treatment: Solutions of selected neurotoxicants were applied to the cells 2 h after seeding. Cells were then returned to the incubator, and hN2^{TM} morphology was then examined at various time points post-seeding. Guidance for concentration ranges used was based on previously published works (Table 2).

Immunocytochemistry: Cell cultures were fixed in situ with 100 µL of a warm (37°C) solution of 8 % paraformaldehyde (PFA) / 8 % sucrose and 0.1% Hoechst 33258 dye in PBS for 20 min. This fixation method effectively preserved the fine morphological features of the hN2^{TM} cultures. Fixative was removed and cells were washed. Cells were then labeled with βIII-tubulin followed by DyLight® 488-conjugated rabbit anti-mouse IgG secondary antibody. Cells were stored at 4°C prior to image acquisition and analysis. Plates were then loaded into a Cellomics ArrayScan VTI HCS reader high-content imaging system (ThermoFisher Scientific, Waltham, MA) for automated image acquisition and morphometric analyses.

Results

Five compounds with known effects on neurite outgrowth in primary rodent neural cultures and well defined molecular mechanisms of action were selected for study (Table 2). At 16 hours of exposure, treatment with 10 µM Bis1, a protein kinase C (PKC) inhibitor, resulted in a 22.3 % decrease in the average number of neurites (control: 1.14 ± 0.22, treated: 0.97 ± 0.13; $p < 0.05$) as well as a 26 % decrease in total neurite length per neuron (control: 38.8 ± 11.8 µm, treated: 31.9 ± 4.5 µm; $p < 0.05$; Table 2). Decreases in hN2^{TM} neurite outgrowth were also observed following treatment with mitogen-activated protein kinase / extracellular-regulated kinase (MEK) signaling inhibitor U0126; glycogen synthase kinase 3-beta (GSK3β) inhibitor lithium chloride (LiCl); the ER-to-Golgi trafficking inhibitor brefeldin A; and the broad spectrum phosphatase inhibitor Na_3VO_4 (Table 3).

These data indicate that neurite outgrowth in hN2^{TM} cells was also inhibited by chemical agents known to affect primary rodent neural culture models (Tables 2 and 3). Neurite outgrowth occurs through a number of interconnected cellular processes, including vesicular and protein trafficking, cytoskeletal reorganization, and intracellular signaling pathways (Larsson, 2006; Yoshimura, et al., 2006; Tang, 2001; Dent & Gertler, 2003; Ditlevsen, et al., 2008). Chemical inhibitors of these processes disrupt neurite outgrowth in both primary rodent and *in vitro* human models, indicating conservation of the molecular mechanisms that underlie neurite outgrowth. These results also suggest that hN2^{TM} cell cultures can act as an *in vitro* system for mechanism based screening of neurotoxicants.

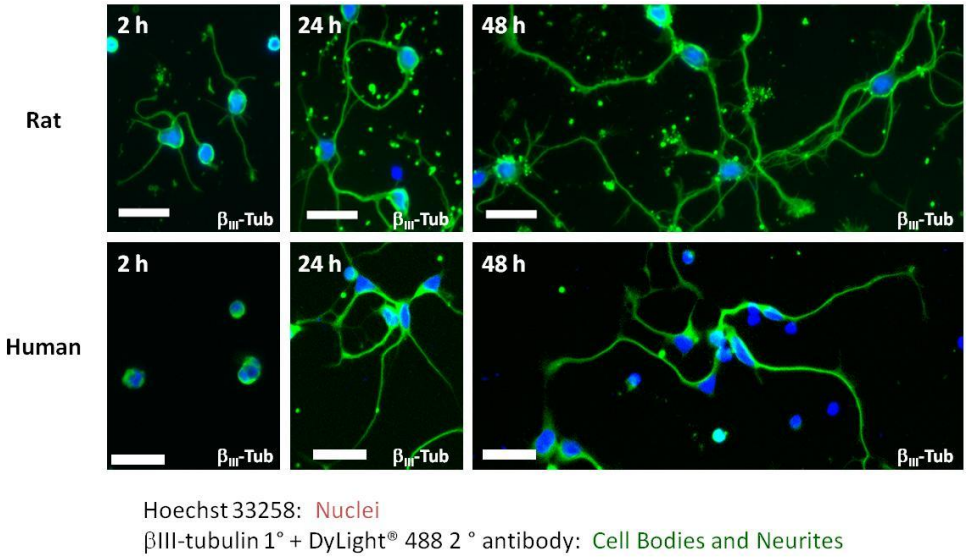

Figure 2. Neurite outgrowth in hN2^{TM} cells and rat cortical neurons cultured for 2-48 hours. Indirect immunofluorescent staining of βIII-tubulin is used to outline cell bodies and neurites for measurement and analysis of outgrowth under experimental conditions. Cells are counterstained with the nuclear dye Hoechst 33258.

Compound	Study	Cell type	Concentration range	Effects
Bis1	Radio et al.	CGC (PND7)[a,b]	1 nM–100 μM	↓ in neurite outgrowth
U0126	Radio et al.	CGC (PND7)	1 nM–100 μM	↓ in neurite outgrowth
LiCl	Takahashi et al.	Hippocampal (E18)[c]	2–15 mM	↓ ratio of axon length to cell body diameter
	Munoz-Montano et al.	CGC (PND7)	1–20 mM	Biphasic ↑ then ↓ in % cells with long neurites
	Hollander and Bennett	DRG (E8-9)[d]	25 mM	↓ in neurite length
Na_3VO_4	Mandel and Banker	Hippocampal (E18)	25–100 μM	↓ length of longest neurite
Bref A	Jareb and Banker	Hippocampal (E18)	0.14–3.57 μM	↓ in % of neurons with axons

[a] PND*x* = post-natal day *x*.
[b] CGC = cerebellar granule cells.
[c] E*x* = embryonic day *x*; designates the age of mouse or rat pups at the time of culture.
[d] DRG = dorsal root ganglia.

Table 2. Chemicals with evidence of neurite outgrowth inhibition in rodent primary neural cultures.

Chemical	Concentration range	Lowest effective concentration[a]			
		ATP content	Neuron density[b]	Average # of neurites per neuron	Total neurite length per neuron
Brefeldin A	0.01–1 μM	0.1	0.1	0.1	0.1
Bis1	0.1–10 μM	n.d.	n.d.	10	10
U0126	0.3–30 μM	30	3	10	10
Na_3VO_4	1–100 μM	30	10	3	3
LiCl	300–30,000 μM	10,000	10,000	10,000	10,000

[a] Values are the lowest effective concentration (in μM) for which a significant change from control was detected using a Dunnett's many-to-one mean contrast test ($p < 0.01$). n.d. = no change detected.
[b] Average number of neurons per field.

Table 3. Summary of concentration-response data.

SUMMARY

In conclusion, human neural progenitors have a strong potential for use as cell-based biosensors for environmental toxins. In this report, we demonstrate that we have (1) optimized methods to genetically modify hNP1™ cells, (2) devised strategies for enriching the neural progenitor cell population using cell surface markers, and (3) developed methods for directed dopaminergic differentiation using defined medium conditions – all towards the goal of accelerating neuronal differentiation for biosensor development. Isolating single-cell colonies for genetically modified human neural progenitor cells has proven to be a challenge. Our recent studies using ROCK inhibitors present a potential solution to this challenge. Continued development of methods to differentiate progenitors into multiple neuronal subtypes and promote long-term cell survival will greatly enhance the sensitivity and robustness of the resulting biosensor.

Culturing long-term, active neural networks on microelectrode arrays (MEAs) has proven difficult. Moreover, assays using MEAs are low throughput, since the arrays have to be cultured individually, making it difficult to monitor and test multiple conditions. As a result, we have also begun an exploration of fluorescence-based assays as a new direction for 'sensor element' development, using neurite outgrowth as a test platform for screening neurotoxicants. Future work will assess other fluorescence-based assays as candidate 'sensor' elements. Such exploration of alternate 'sensor elements' will broaden the utility of a human neural cell-based biosensor.

RECENT PUBLICATIONS & PATENTS

Harrill J.A. T. M. Freudenrich, D.W. Machacek, S.L. Stice, W.R. Mundy. 2010. Quantitative assessment of neurite outgrowth in human embryonic stem cell derived hN2™cells using automated high-content image analysis. *Neurotoxicology*, **31** (3):277-90. *[Collaboration with EPA]*

Patent Disclosure on neural markers and dopaminergic cells to the University of Georgia.

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Aruna Biomedical

Job #7015	DNR DoD Biosensor			**Cost:**	**899,084.00**	
Agency	ONR			**Fee:**	**62,916.00**	
Phase	II			**Amount:**	**962,000.00**	
Contract #	N00014-10-C-0066					
Period of Performance:	12/11/09					
	12/11/10					

		April - June '10	Jan- Mar '10	2010 Year To Date	Dec '09	Project To Date	Estimate to Complete
DIRECT LABOR		$18,710.58	$32,009.76	$50,720.34	$7,054.07	$57,774.41	$162,872.23
DIRECT LABOR FRINGE	22.97%	$4,297.81	$7,352.64	$11,650.45	$1,620.32	$13,270.77	$37,412.23
DIRECT LABOR OVH	46.14%	$10,616.08	$18,161.81	$28,777.89	$4,002.36	$32,780.25	$92,411.44
CONTRACT LABOR		$0.00	$0.00	$0.00	$0.00	$0.00	$0.00
TOTAL LABOR & OVH		$33,624.47	$57,524.21	$91,148.68	$12,676.75	$103,825.43	$292,695.90
CONSULTANTS		$0.00	$0.00	$0.00	$0.00	$0.00	$0.00
EQUIPMENT		$0.00	$0.00	$0.00	$0.00	$0.00	$0.00
MATERIAL & PARTS		$29,294.03	$28,996.54	$58,290.57	$1,649.94	$59,940.51	$236,342.49
OTHER DIRECT COSTS		$0.00	$538.44	$538.44	$0.00	$538.44	$11,161.56
SUBCONTRACTS		$0.00	$0.00	$0.00	$0.00	$0.00	$0.00
TRAVEL		$0.00	$0.00	$0.00	$0.00	$0.00	$5,000.00
TOTAL OTHER DIR. COST		$29,294.03	$29,534.98	$58,829.01	$1,649.94	$60,478.95	$252,504.05
TOTAL COSTS BEFORE G&A		$62,918.50	$87,059.19	$149,977.69	$14,326.69	$164,304.48	$545,199.95
GEN. & ADMIN. (G&A)	27.78%	$17,478.76	$24,185.04	$41,663.80	$3,979.96	$45,643.76	$143,935.91
TOTAL COST		$80,397.26	$111,244.23	$191,641.49	$18,306.65	$209,948.14	$689,135.86
FEE/PROFIT	7.00%	$5,627.81	$7,785.34	$13,413.15	$1,281.18	$14,694.32	$48,221.68
REVENUE		$86,025.07	$119,029.57	$205,054.64	$19,587.82	$224,642.46	$737,357.54

Estimate of Costs to Complete *$737,357.54*